Identification and characterisation of a *Bacillus licheniformis* strain with profound keratinase activity for degradation of melanised feather

Emeka A. Okoroma[1], Hemda Garelick[1], Oduola O. Abiola[2] and Diane Purchase[1*]

[1]Department of Natural Sciences, Middlesex University, The Burroughs, London NW4 4BT

[2]PAP Rashidah Sa'adatul Bolkiah Institute of Health Sciences, Universiti Brunei Darussalam, Gadong, Brunei Darussalam, BE 1410

*Corresponding author

Email addresses:

EAO: e.okoroma@mdx.ac.uk

HG: h.garelick@mdx.ac.uk

OOA: oduola.abiola@ubd.edu.bn

DP: d.purchase@mdx.ac.uk

---

[1]Department of Natural Sciences, School of Health and Social Sciences, Middlesex University, The Burroughs, London NW4 4BT, U.K.

Tel: +44 (0)2084115262; Fax: +44 (0)2084116774; email: d.purchase@mdx.ac.uk

Abstract

Significant amount of keratins in the form of feather, hair, hoof and horn are generated annually by the livestock industry. Keratinases are increasingly important in the reprocessing and environmental pollution control of keratin wastes. The aim of this study is to isolate a microbial strain of high keratinase activity and to evaluate its feather degrading potential. Thirty-two keratin degrading microbial strains from farmyard wastes and primary effluent were isolated using a selective medium containing feather meal at 30, 37 and 50 °C. One of the isolates, which demonstrated the highest keratinolytic activity (11.00 ± 0.71 Uml^{-1}) was identified as a species of *Bacillus licheniformis* based on the 16S rDNA analysis, designated as strain N22 and deposited in a culture collection. Optimum keratinase production by this bacterium was achieved in 32 h using a minimum growth medium containing 1.1% (w/v) feather meal at 50 °C and pH 8.5. The molecular weight of the keratinase was ≈ 28 KDa as determined sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The keratinase reported here significantly degraded melanised feather in 48 h in the absence of reducing agents. There are few reports on the evaluation of feather degrading ability of keratinases using highly resistant melanised feather. The efficient degradation of melanised feathers by this keratinase may offer an environmentally friendly solution to the degradation of feather waste and other organic matter of similar molecular composition.

Key words: *Bacillus*, Keratinase, Degradation, Keratin, Feather, Melanised feathers.

1. Introduction

The poultry industry produces a significant amount of feather waste; the United States and the United Kingdom generate about 4 billion pounds (Schmidt 1998) and 150,000 tonnes (331 million pounds; United Kingdom Food and Drink Processing Mass Balance 2004) of feather waste per annum respectively. The disposal of such considerable quantities of waste by landfill requires large areas of land and the alternative option of incineration creates additional pollution burden. Neither method is economical or environmentally sustainable. In addition, feather wastes are potentially valuable raw materials for the production of nutrient-rich animal feed supplements and amino acids (Shih 1993; Onifade et al. 1998). Feather waste processing methods such as steam pressure, chemical treatment and feather milling are cost and labour intensive and may reduce the product's nutritional value by destroying important amino acids (Shih 1993; Wang and Parsons 1997).

Feather is composed of approximately 90% pure keratin; predominantly in the form of β-keratin (Fraser et al. 1971). The tertiary structure of β-keratin is highly organised: the cross-linking of disulfide and hydrogen bonds along with hydrophobic interactions stabilises the structure and together with its aggregated amyloid-like fibrillar form confer mechanical strength as well as chemical and enzymatic resistance (Alexander and Hudson 1954; Fuchs 1995; Parry and North 1998). Interestingly, despite these highly stabilising characteristics, an increasing number of enzymes, isolated from certain environmental microbial strains are known to degrade feather keratin (Noval and Nickerson 1959; Riffel et al. 2003; Gunderson 2008). Enzymatic degradation of feather is particularly important because it provides an operational advantage over bacterial fermentation that requires controlled conditions for cell growth (Ramnani and Gupta 2007).

Keratinases efficiently hydrolyse keratin and degrade feathers into useful livestock feeds (Chandrasekaran and Dhar 1986; Onifade et al. 1998; Odetallah et al. 2003) and organic fertilizers (Choi and Nelson 1996). In addition, they are useful for removing hair from hides in the leather processing industry (Mukhopadyay and Chandra 1993; Macedo et al. 2005; Tiwary and Gupta 2010) and for the degradation of prions-the causative agent of prion diseases (Shih 2002; Langeveld et al. 2003). Thus they have important applications in biotechnology and feather waste management.

A number of keratinase-producing microorganisms e.g. fungi (Gradisar et al. 2005; Ismail et al. 2012), actinomycetes (Bressollier et al. 1999), *Streptomyces* (Tapia and Simoes 2008), *Bacillus* (Williams et al. 1990) and *Pseudomonas* (Sharma and Gupta 2010) species have been isolated from various environmental sources such as soil; poultry farm wastes and raw feather. The great potential for keratinase in the biotechnology industry demands that the most efficient and environmentally friendly sources are identified and exploited for maximum production. Farmyard wastes are characterised by a rich and diversified microbial ecology (Tapia and Simoes 2008; Tork et al. 2010, Riffel and Brandelli 2006). Thus they constitute an excellent source of keratinase producing microorganisms. In the present work, we have isolated a prolific keratinase producing bacterium from farmyard waste and designated it as *Bacillus licheniformis* N22. The keratinase is able to significantly degrade melanised feather and may have potential to degrade other keratinised organic matter more efficiently than currently known.

2. Materials and Methods

2.1. Isolation and identification of microorganisms

Minimum Growth Medium (MGM) containing (in gl^{-1}): NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4.7H_2O$, 0.1; pH 7 (Wang and Shih 1999) was used as the basic growth medium.

Microbial strains were isolated from primary effluent (Deepham Sewage Treatment Facility, Thames Water, Edmonton, North London, UK) and poultry/animal wastes (A.K Woods Poultry Farm, Fold Farm Partners and Leamon Pig Farm Ltd UK). Sample of wastes (1 g) were serially diluted in a quarter-strength Ringer's solution (Oxoid UK), plated out on feather meal agar (10% commercial feather meal (Chettles Ltd UK) in MGM and 1% agar) plates and incubated at 30 °C, 37 °C or 50 °C for 24, 48 and 72 h. Single colonies were passaged twice on feather meal agar (FMA) plates to obtain microbial monoculture. The purified isolates were grown in Nutrient Broth (Oxoid UK) and stored in liquid nitrogen in 0.5 ml aliquots.

2.2. Screening for proteolytic and keratinolytic activity

A casein agar assay was designed to screen for proteolytic activity; this method is based on the procedures described by Lin et al (1992) and Tork et al. 2010. Briefly, a number of wells were aseptically punched onto a casein agar (Oxoid UK) plate surface, using a sterile 7 mm diameter puncher. The wells were inoculated with 100 µl of the bacterial culture and incubated at 30 °C, 37 °C or 50 °C for 24 h. The diameters of the hydrolysis rings produced by the isolates were measured; those that produced the highest diameters were selected for further examination. A validation experiment was carried out using the crude enzyme extract at 1:0; 1:10; 1:100; and 1:1000 dilutions.

Strains selected for high activity were further cultured in 50 ml MGM containing 1.1% feather meal. The crude enzyme extract was recovered by centrifugation at x 4750 *g* for 20 min in a Rotina 420R Centrifuge (Hettich Germany). The supernatant was collected and vacuum filtered through 0.45 µm sterile membranes (Pall Corporation USA).

Keratinase activities of the crude enzyme extracts were determined using the method described by Letourneau et al. (1998). One unit of keratinase activity (U) was defined as the

amount of enzyme producing an absorbance change of 0.01 units (A_{595}). The keratin azure substrate was treated prior to the assay to ensure uniformed release of the azure dye and reliable absorbance measurement. Briefly, keratin azure was incubated with 5 times the displaceable volume of distilled water for 1 h at 50 °C and 250 rpm in a rotary incubator. The resulting keratin azure was washed thrice with sterile distilled water by shaking vigorously for 1 min and allowed to dry at 30 °C in an oven.

Initial identification of the microorganism was carried out based on their morphological, physiological and biochemical characteristics (Holt 1993). This consisted of colony morphology, Gram staining, endospore test, anaerobic growth test, motility test, oxidase test, catalase test, Triple Sugar Iron Test (TSI), nitrate to nitrite reduction, Voges-Proskauer (VP) Test, indole production, arginine hydrolase test, Urea hydrolysis test and the effect of salt concentration and pH on N22 growth. In addition, molecular identification using MicroSeq™ based on the 16S rDNA full gene sequence was performed by the National Collections of Industrial Food and Marine Bacteria (NCIMB), UK.

To generate the microbial mass spectral profile, a single bacterial colony was selected and the whole cell protein was extracted with 1 ml mixture of acetonitrile and 70 % formic acid (1:1 v/v). The extracted protein sample (1µl) was conjugated with 1µL of the α-cyano-4-hydroxycinnamic acid matrix (Sigma, USA) and analysed by the MALDI-TOF MS (AXIMA CFR, Shimadzu Biotech).

2.3. Keratinase production and characterisation

Keratinase production was optimised using the following parameters: temperatures at 37 °C or 50 °C; pH 7, 8.5 or 10) and feather meal concentration of 0.8%, 1.1% or 1.4% (w/v). All experiments were carried out in triplicates.

Cell-free crude keratinase extract was concentrated using a Centriconplus-70 centrifugal filter device (Millipore Inc.) with a 10 KDa molecular weight cut-off and purified by affinity chromatography using a HiTrap Blue HP purification column (GE Healthcare Bio-sciences). Concentrated crude extract was applied to the column at a rate of 0.5 - 1 ml/min. Binding buffer (50 mM KH_2PO_4, pH 7.0) was applied until eluent became clear, the column was eluted with the elution buffer (50 mM KH_2PO_4, 1.5 M KCl, pH 7.0) and 5 ml fraction (EF) was collected. To determine the stability of the purified keratinase, samples were stored at 4 °C and its activity reassessed after 8 weeks.

The molecular weight of the purified keratinase was also determined by SDS-PAGE using a 10% gel, and traced against standard molecular weight markers (15 KDa-120 KDa).This was confirmed by MALDI-TOF MS where the purified keratinase sample (1 µl) was conjugated to sinapinic acid matrix (1 µl) on a sample metal plate and allowed to crystallise at room temperature and analysed.

2.4. Degradation of melanised feathers

Melanised feathers were washed with sterile distilled water and tested for microbial contamination. These were dried (50 °C) to obtain dry weight, suspended in distilled water and incubated at 50 °C with crude or denatured crude keratinase for 48 hours in a rotary incubator (Stuart, UK) at 250 rpm. The differences in weights indicated the extent of degradation of the feather substrates. The data were analysed using a two-sample t-test for their significance at the 95% confidence interval using Minitab® 15.1.

3. Results and Discussion

3.1. Isolation and identification of feather degrading bacteria

Since the first report on the isolation of extracellular alkaline serine protease from *Bacillus* sp. strain 221 (Horikoshi 1971), the search for industrially relevant proteases from different environmental sources such as farmyard wastes (Tork et al. 2010) and sewage sludge (Semple et al. 2001) has been continuous.

Microbial strains from primary effluent, poultry and animal wastes were isolated on feather meal agar (FMA) plates. The proteolytic and keratinase activities of the isolates were determined by the casein agar assay. This assay is based on the procedures described by Lin et al. (1992) and Tork et al. (2010) where the diameter of the clear zone was used to determine the microbial growth rate.

In an experiment to demonstrate the usefulness of the hydrolysis ring assay, the casein ring diameters for the neat, 1:10; 1:100; 1:1000 dilutions of crude enzyme were measured as 20 mm, 16.3 mm, 12.5 mm and 6.3 mm respectively (Fig. 1).The casein ring diameter was inversely correlated to the dilution factor of the crude keratinase (Pearson correlation = 0.761, p=0.239). In this study a correlation between the sizes of the hydrolysis rings and the protease catalytic activity was demonstrated (Fig. 1), which establishes the hydrolysis ring assay as an efficient, cost and labour effective semi-quantitative method for measuring proteolytic activities. Based on the casein ring analysis, thirty-two best performing microbial strains were isolated for further study.

Casein-agar assay is a useful tool for the screening of proteolytic microorganisms and determination of proteolytic and keratinolytic activities. Casein is a highly stable and hydrophobic non-fibrous protein which does not coagulate or easily denatured by heat but can

be hydrolysed by certain proteases (Fujiwara et al. 1993; Cheng et al. 1995). These characteristics informed our choice of the casein hydrolysis ring assay for the identification of proteolytic microorganisms that can hydrolyse casein and other structurally similar substrates such as keratins.

It is important to note that since not all proteases can degrade keratin, an evaluation of keratinolytic activity of potential keratinase producers should be confirmed by the keratin azure assay which is highly specific for determination of keratinase activity. Of the thirty-two isolates, strain N22 demonstrated the highest keratinase activity (11.00 ± 0.71 Uml^{-1}; Fig. 2) and was selected for further study. N22 keratinase was found to express 58% higher activity than the well-characterised *B.licheniformis* PWD-1 (ATCC 53757) keratinase.

Isolate N22 was identified as a Gram-positive, endospore forming, mostly alkalophilic, mildly thermophilic and halotolerant bacterium displaying a number of characteristics similar to the *Bacillus* species listed in the Bergey's Manual of Determinative Bacteriology (Holt 1993; Table 1). The aerobic, mild and easily manageable growth conditions (37-60 °C and pH 6-10) make this bacterium an attractive candidate for biotechnological applications.

Gene sequence analysis of the 16S rDNA using MicroSeq™ indicated that strain N22 is closely related to *B. licheniformis* which are known to be prolific producers of keratinases (Williams et al. 1990; Manczinger et al. 2003). Strain N22 has 99.93% homology with *B. licheniformis* ATCC14580. Strain N22 deviates from the well characterised PWD-1 as the former is unable to ferment lactose. Furthermore, characteristic peaks of the MALDI-TOF mass spectral fingerprints differ for the two strains (data not shown). Also, the crude keratinase produced by strain N22 expressed 58% higher activity than the keratinase from PWD-1 on keratin azure substrate (Fig. 2). These confirm that strain N22 is a different

organism. Strain N22 has been deposited with the National Collection of Industrial Food and Marine Bacteria (NCIMB): its ascension number is NCIMB 41708.

3.2. Optimisation of keratinase production and characterisation of keratinase

The interplay of various factors such as temperature, pH, substrate concentration, the nature and composition of carbon and nitrogen sources as well as the condition of inoculants, influences cell growth and survival, and the level of protease synthesis in a microbial culture (Singh et al. 1975). Thus, optimisation of these factors is important for an effective synthesis of biological entities such as keratinases.

Keratinase production by *B. licheniformis* N22 occurred over a wide pH range (6-12). The optimum production of keratinase was achieved at a substrate (feather meal) concentration of 1.1%, pH 8.5 and 50 °C over 32 h. Keratinase production was greatly inhibited at acidic pH (pH ≤4), substrate concentration above 1.1% and a temperature of 65 °C and above (Fig. 3). Maximum keratinase activity of 11 ± 0.71Uml^{-1} was attained at pH 8.5, feather meal concentration of 1.1% (w/v) and a temperature of 50 °C. The most important limiting factors affecting keratinase synthesis appeared to be low pH (pH≤4) and high temperature (≥65 °C). As *B. licheniformis* is mildly thermo-tolerant with its optimum temperature of 50 °C, it may be adapted for microbial composting of organic wastes.

Feather meal substrate induced keratinase synthesis by *B. licheniformis* N22 and resulted in a 68% increase compared to culture grown in Nutrient Broth (results not shown). This confirms that high substrate concentration reduced keratinase production (Fig. 3) as previously reported by Brandelli and Riffel (2005), Wang and Shih (1999) and Lin and Yin (2010). As approximately 40% of total cost of enzyme production is due to the cost of growth substrates (Kumar and Parrack 2003); the use of low cost and readily available feather meal or chicken

feather substrate will enable the sustainable production of strain N22 keratinase on an industrial scale. In addition, the use of feather waste as growth substrates for keratinase producing microorganisms will invariably serve as one of a most efficient means of managing the significant amounts of chicken feather wastes produced by the poultry processing industry (American Chemical Society 2011).

B. licheniformis N22 keratinase was purified by affinity chromatography and the molecular weight determined by SDS-PAGE analysis and MALDI TOF-MS was found to be ≈ 28 KDa. The single mass peak (Fig. 4a) and single band (Fig. 4b) obtained indicates that the purified keratinase is homogeneous and monomeric (has no subunits).

The activity of the purified keratinase was maintained at 11 Uml^{-1} after 56 days storage at 4 °C compared to the keratinase produced by *B. licheniformis* PWD-1 which had been reported to lose a quarter of its activity after 19 days when stored at the same temperature (Shih and William 1992). Keratinase from *Streptomyces fradiae* also retained its activity for several weeks at 4 °C when stored at pH 7 but rapidly loses its activity at pH 8.5 (Nickerson and Noval 1961).

3.3 Degradation of melanised feather

B. licheniformis N22 crude keratinase was investigated for its catalytic activity on melanised feather. Melanised feather was significantly degraded by the cell-free crude keratinase (Fig. 5). A mean reduction of about 80% in the weight of melanised feather (52 ± 2 mg to 10 ± 3 mg) was obtained when digested with crude extract for 48 h as compared to 12% (52 ± 2 mg to 46 ± 3 mg) reduction for control experiment in which the crude keratinase had been boiled at 100 °C for 30 minutes to denature it ($P = 0.038$). The residue (mainly rachises) was soft and brittle and had to be handled with great care during drying and weighing. The rachis

constitutes most of the total feather mass and has been reported to resist degradation by *B. licheniformis* (Ramnani et al. 2005).

In the present study, melanised feathers were cleaned by agitation with sterile distilled water to ensure that they remained structurally uncompromised and free from microbial contamination (Cortezi 2008). Sterilisation by autoclaving denatures feather keratin and causes the feather to be susceptible to proteolytic attack as they lose their insolubility and resistance to enzymatic degradation (Suzuki et al. 2006; Gunderson et al. 2008).

There are relatively few reports on the evaluation of keratinolytic activity using melanised feather substrate. Rather, in most reported feather degradation studies, the feather substrates are usually young, downy white feathers (Brandelli and Riffel 2005; Cao et al. 2008; Zhang et al. 2009). Notably, melanised feathers are generally more resistant to enzymatic attack than white feathers due to the presence of the pigment melanin that binds to and inhibits keratinases (Goldstein et al. 2004; Gunderson et al. 2008) as well as other enzymes (Kuo and Alexander 1967). Our study has shown that at optimum conditions the reported enzyme demonstrated a much more profound catalytic activity than any of the known keratinases.

Feather degradation by keratinases has been mostly performed in the presence of suitable reducing agents such as live cells or chemical reductants (Brandelli and Riffel 2005; Ramnani and Gupta 2007; Liang et al. 2010). Cell-free keratinases of *Streptomyces* BA_7 (Korkmaz et al. 2003), *Streptomyces* S7 (Tatineni et al. 2008) and *B. licheniformis* ER-15 (Tiwary and Gupta 2010) have been reported to degrade feather in 24 h, 97 h and 12 h respectively. However, only 10-20% degradation is reportedly achieved by cell-free keratinases in the absence of reductants (Hossam et al. 2007).

Most purified keratinases are unable to effectively degrade native keratin for reasons which include: (a) the high degree of disulfide bonds in the keratin molecules (Bockle et al. 1995; Riffel et al. 2007) and (b) the removal, during purification, of fermentation culture constituents that are capable of reducing or breaking disulphide bonds (Cao et al. 2008). The latter suggests that the presence of a consortium of enzymes may be required to enhance feather keratin degradation (Sharma and Gupta 2010). In contrast, the keratinase of *B. licheniformis* N22 was able to significantly degrade melanised feather in the absence of reducing agents. Feather degradation generally occurs under alkaline conditions as a result of deamination (Kunert 1992); this is within the optimum pH range for the keratinase reported in this study. Thus it arguably has a more robust potential for biotechnology exploitation especially in feather waste management.

Conclusions

The keratinase reported here is stable when stored in liquid nitrogen (-196 °C) and able to significantly degraded highly resistant melanised feather in 48 h in the absence of reducing agents; it offers an environmentally friendly solution to manage keratin and feather waste. Further investigation on its ability to biodegrade other recalcitrant materials may widen its potential application.

Acknowledgements

This work was supported by the Alberta Prion Research Institute Canada and the Institute of Social and Health Research, Middlesex University, London, UK.

References

Alexander, P., Hudson, R. F., 1954. Wool, its chemistry and physics. Reinhold Publishing Co, New York, pp. 354-356.

American Chemical Society, 2011. Advance towards making biodegradable plastics from waste chicken feathers, ScienceDaily, Online at [http://www.sciencedaily.com/releases/2011/03/110331142204.htm] Last accessed: 19/04/12.

Bockle, B., Galunsky, B., Muller, R., 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces*pactum DSM 40530. Applied Environmental Microbiology61, 3705-3710.

Brandelli, A., Riffel, A., 2005. Production of an extracellular keratinase from *Chryseobacterium* sp growing on raw feathers. Electronic Journal of Biotechnology 8, 35-42.

Bressollier, P., Letourneau, F., Soussottee, V., Urdaci, M., Verneuil, B., 1999. Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. Applied and Environmental Microbiology 65, 2570-2576.

Cao, L., Tan, H., Liu, Y., Xue, X., Zhou, S., 2008. Characterization of new keratinolytic *Trichderma atroviride* strain F6 that completely degrades native chicken feather. Letters in Applied Microbiology 46, 389-394.

Chandrasekaran, S., Dhar, S.C., 1986.Utilization of multiple Proteinase concentrate to improve the nutritive value of chicken feather meal. Journal of Leather Research.4, 23-30.

Cheng, S.W., Hu, H.M., Shen, S.W., Takagi, H., Asano, M., Tsai, Y.C., 1995. Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. Bioscience Biotechnology and Biochemistry 59, 2239-2243.

Choi, J.M., Nelson, P.V., 1996. Developing a slow-release nitrogen fertilizer from organic sources. 2. Using poultry feathers. Journal of the American Society for Horticultural Science 121, 634-638.

Cortezi, M., Contiero, J., de Lima, C.J.B., Lovaglio, R.B., Monti, R., 2008. Characterization of a feather degrading *Bacillus amyloliquefaciens* protease: A New Strain. World Journal of Agricultural Science 4, 648-656.

Fraser, R.D.B., MacRae, T.P., Parry, D.A.D., Suzuki, E., 1971. The structure of feather keratins. Polymer 12, 35-6.

Fuchs, E., 1995.Keratins and the skin. Annual Review of Cell and Developmental Biology 11, 123-153.

Fujiwara, N., Masui, A.T., Imanaka, T., 1993. Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. Journal of Biotechnology 30, 245-256.

Goldstein, G., Flory, K.R., Browne, B.A., Majid, S., Ichida, J.M., Burtt, E.H., 2004. Bacterial degradation of black and white feathers. The American Ornithologists' Union 121, 656-659.

Gradisar, H., Friedrich, J., Krizaj, I., Jerala, R., 2005. Similarities and specificities of fungal keratinolytic proteases: Comparison of keratinases of *Paecilomyces marquandii* and *Doratomycesmicrosporus* to some known proteases. Applied and Environmental Microbiology 71, 3420-3426.

Gunderson, A.R., 2008. Feather-degrading bacteria: A new frontier in avian and host- parasite research? The American Ornithologists' Union 125, 972-979.

Gunderson, A.R., Frame, A.M., Swaddle, J. P., Forsyth, M. H., 2008. Resistance of melanized feathers to bacterial degradation: Is it really so black and white. Journal of Avian Biology,39,539–545.

Holt JG (Ed.), 1993. Bergey's Manual of Systematic Determinative Bacteriology 9th Edition Williams and Wilkins, Baltimoore, pp. 559-561.

Horikoshi K., 1971. Production of alkaline enzymes by alkalophilic microorganisms. I. Alkaline protease produced by *Bacillus* no. 221. Agricultural and Biological Chemistry 35, 1407-1414.

Hossam, M.S., Azad, A.K., Abu sayam, S.M.,,Mostafa, G., Hoq, M.M., 2007. Production and partial characterisation of feather-degrading keratinolytic serine protease from *Bacillus licheniformis* MZK-3. Journal of Biological Sciences 7, 599-606.

Ismail, A-M. S., Housseiny M.M., Abo-Elmagd, H.I., El-Sayed N.H., Habib M., 2012. Novel keratinase from *Trichoderma harzianum* MH-20 exhibiting remarkable dehairing capabilities. International Biodeterioration and Biodegradation 70, 14-19.

Kumar, G., Parrack, P., 2003. Arrowroot (*Maranthaarundinacea*) starch as a new low-cost substrate for alkaline protease production. World Journal of Microbiology and Biotechnology 19, 757-762.

Korkmaz, H., Unaldi, M.N., Aslan, B., Coral, G., Arikan, B., Dincer, S., Colak, O., 2003.Keratinolytic activity of *Streptomyces* strain BA7, a new isolate from Turkey. Annals of Microbiology 53, 85-93.

Kunert, J. 1992. Effect of reducingagents on proteolytic and keratinolyticactivity of enzymes of *Microsporumgypseum*. Mycoses 35, 343-348.

Kuo, M.-J., Alexander, M., 1967. Inhibition of the lysis of fungi by melanins. Journal of Bacteriology 94, 624-629.

Langeveld, J.P.M., Wang, J.J., Van De Wiel, D.F.M., Shih, G.C., Garssen, J., Bossers, A., Shih, J.C.H., 2003. Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. Journal of Infectious Diseases 188, 1782-1789.

Letourneau, F., Soussotte, V., Bressollier, P., Branland, P., Verneuil, B., 1998.Keratinolytic activity of *Streptomyces sp*. S.K1-02: a new isolated strain. Letters in Applied Microbiology 26, 77-80.

Liang, X., Bian, Y., Tang, X-F., Xiao, G., Tang, B., 2010. Enhancement of keratinolytic activity of a thermophilicsubtiliase by improving autolytic resistance and thermostability under reducing conditions. Applied Microbiology and Biotechnology 87, 999-1006.

Lin, H.-H., Yin, L-J., 2010. Feather meal and rice husk enhanced keratinase production by *Bacillus licheniformis* YJ4 and characteristics of produced keratinases. Journal of Marine Science and Technology 18, 458-465.

Lin, X., Lee, C.G., Casale, S.E., Shih, J.C.H., 1992.Purification and characterisation of a keratinase from a feather-degrading *Bacillus licheniformis*. Applied Environmental Microbiology 58, 3271-3275.

Macedo, A.J., Beys da Silva, W.O., Gava, R., Driemeier, D., Henriques, J.A.P., Termignoni, C., 2005. Novel Keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. Applied and Environmental Microbiology 71, 594–596.

Manczinger, L., Rozs, M., Vagvolgyi, C., Kevei, F., 2003. Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World Journal of Microbiology and Biotechnology 19, 35-39.

Mukhopadyay, R.P., Chandra, A.L., 1993. Protease of keratinolytic *Streptomycete* to unhair goat skin. Indian Journal of Experimental Biology 31, 557-558.

Nickerson W.J., Noval J.J.: *Process of treating keratinous material and a keratinase produced thereby.* US patent 2988487 1961.

Noval, J.J., Nickerson, W.J., 1959. Decomposition of native keratin by *Streptomyces fradiae*. Journal of Bacteriology 77, 251–263.

Odetallah, N. H., Wang, J.J., Garlich, J.D., Shih, J.C.H., 2003. Keratinase in starter diets improves growth of broilers chicks. Poultry Science 82, 664-670.

Onifade, A.A., Al-Sane, N.A., Al-Musallam, A.A., Al-Zarban, S., 1998: A review: Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresource Technology, 66, 1–11.

Parry, D.A.T., North, A.C.T., 1998. Hard a-keratin intermediate filament chains: substructure of the N- and C-terminal domains and the predicted structure and function of the C-terminal domains of type I and type II chains. Journal of Structural Biology 122**,** 67-75,

Ramnani, P., Gupta, R., 2007. Keratinasesviv-a-vis conventional proteases and feather degradation. World Journal of Microbiology and Biotechnology 23, 1537-1540.

Ramnani, R., Singh, R., Gupta, R., 2005. Keratinolytic potential of *Bacillus licheniformis* RG1: Structural and biochemical mechanism of feather degradation. Canadian Journal of Microbiology 51, 191-196.

Riffel, A., Bellato, C.M., Souza, G.H.M.F., Eberlin, M.N., Tavares, F.C.A., 2007. Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. Kr6. Journal of Biotechnology 128, 693-703.

Riffel, A., Brandelli, A. 2006. Keratinolytic bacteria isolated from feather waste. Brazilian Journal of Microbiology 37, 395-399.

Riffel, A., Lucas, F., Heeb, P., Brandelli, A., 2003. Characterization of a new keratinolytic bacterium that completely degrades Native feather keratin. Archives of Microbiology 179, 258-65.

Schmidt, W.F., 1998. Innovative feather utilization strategies. In: Proceeding, Poultry Waste Management Conference. 19-22 Oct. 1998, Springdale, Arkansas, pp.276-282.

Semple, K.T., Reid, B.J, Fermor, T.R., 2001. Impact of composting strategies on the treatment of soils contaminated with organic pollutants. Environmental Pollution 112, 269-283.

Sharma, R., Gupta, R., 2010. Substrate specificity characterisation of a thermostablekeratinase from *Pseudomonas aeruginosa* KS-1. Journal of Industrial Microbiology and Biotechnology 37, 785-792.

Shih, J.C.H., 1993. Recent developments in poultry waste digestion and feather utilization. Poultry Science 72, 1617-1620.

Shih, J.C.H., 2002. Method and composition for sterilizing surgical instruments. US Patent Aplication US 2002/0192731.

Shih, J.C.H., William, C.M., 1992. Purified *Bacillus licheniformis* PWD-1 keratinase. US Patent US5171682.

Singh, D.P., Sing, R., Vyas, S.R., 1975. Effect of pH, temperature, nitrogen source glucose concentration on acid protease production by *Aspergillus niger* mutant. Indian Journal of Microbiology 15, 109-113.

Suzuki, Y., Tsujimoto, Y., Matsui, H., Watanabe, K., 2006. Decomposition of extremely hard-to-degrade animal proteins by thermophilic bacteria. Journal of Biosciences 102, 73-81.

Tapia, D.M.T., Simoes, M.L.G., 2008. Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater. African Journal of Biotechnology, 7, 296-300.

Tiwary, E., Gupta, R., 2010. Medium optimization for a novel 58 kDa dimerickeratinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. Bioresource Technology 101, 6103–6110.

Tork, S., Aly, M.M., Nawar, L., 2010. Biochemical and molecular characterisation of a new local keratinase producing *Pseudomonas* sp. MS21. Asian Journal of Biotechnology 2, 1-13.

Tatineni, R., Doddapaneni, K.K., Potumarthi, R.V., Vellanki, R. N., Kandathil, M. T., Kolli, N., Mangamoori, L. N., 2008. Purification and characterisation of an alkaline keratinase from *Streptomyces* sp. Bioresource Technology 99, 1596-1602.

United Kingdom Food and Drink Processing Mass Balance, 2004. [http://www.massbalance.org/downloads/projectfiles/2182-00335.pdf] Last accessed 19/04/12.

Wang, J.J., Shih, J.C.H., 1999. Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29. Journal of Industrial Microbiology and Biotechnology 22, 608-616.

Wang, X., Parsons, C.M., 1997. Effect of processing systems on protein quality of feather meal and hog hair meal. Poultry Science 76, 491-496.

Williams, C.M., Ritcher, C.S., MacKenzie Jr., J.M., Shih, J.C.H., 1990. Isolation, identification and characterization of feather-degrading bacterium. Applied and Environmental Microbiology 56, 1509-1515.

Zhang, B., Sun, Z., Jiang, D-D., Niu, T-G., 2009. Isolation and purification of alkaline keratinase from *Bacillus* sp. 50-3. African Journal of Biotechnology 8, 2598-2603.

Table 1

Morphological, physiological and biochemical characterisation of isolate N22

Tests	**Response**
Colony morphology	Rod-shaped, rough edge, mucoid, domed surface, creamy-white colour
Gram stain	+
Endospores formation	+
Anaerobic growth	-
Motility	+
Oxidase reaction	+
Catalase reaction	+
Glucose	+
Lactose	-
Iron	-
Reduction of nitrates to nitrites	+
Voges-Proskauer (VP)	+
Indole production	-
Arginine DiHydrolase	+
Urease	+
Hydrolysis of casein	+
Onset of growth on nutrient agar plate	
(37^{o}C)	9 h
(50^{o}C)	5 h
Growth in nutrient agar at NaCl conc. 2-12 %	+
Growth in nutrient broth at pH 6-12	+

Figures

Fig. 1. Casein agar plate showing the relationship between keratinase activity and the casein hydrolysis rings. Clockwise from top left are inoculations with keratinase dilution at 1:0, 1:10, 1:100 and 1:1000. The casein agar plate was incubated at 50 °C for 24 h.

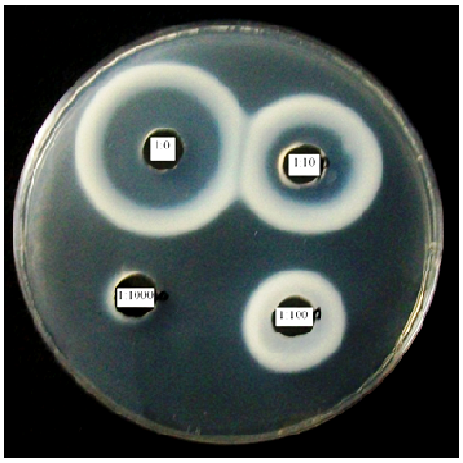


Fig. 2. Keratinase activity (U ml-1 ± SD) for the 32 microbial isolates as measured by the keratin azure assay.

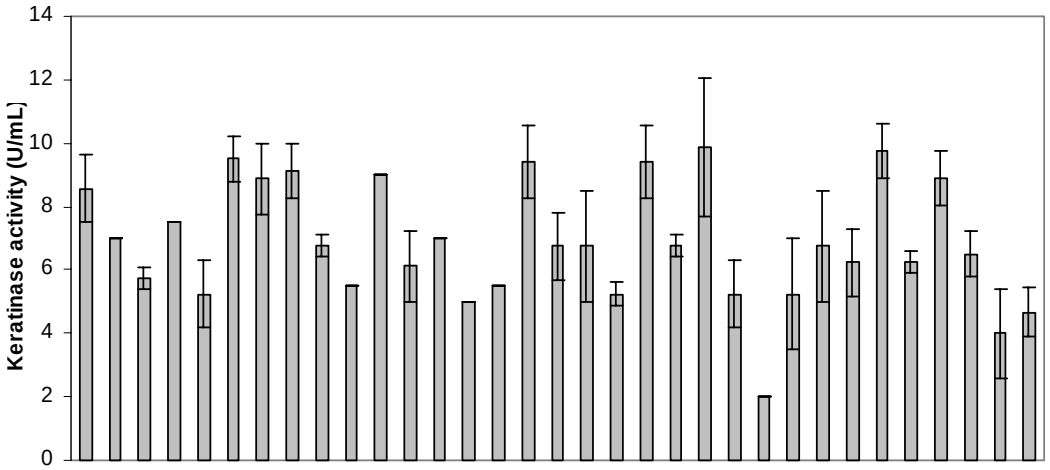


Fig. 3. The effects of temperature, pH and substrate concentration, on keratinase production by *B. licheniformis* N22.

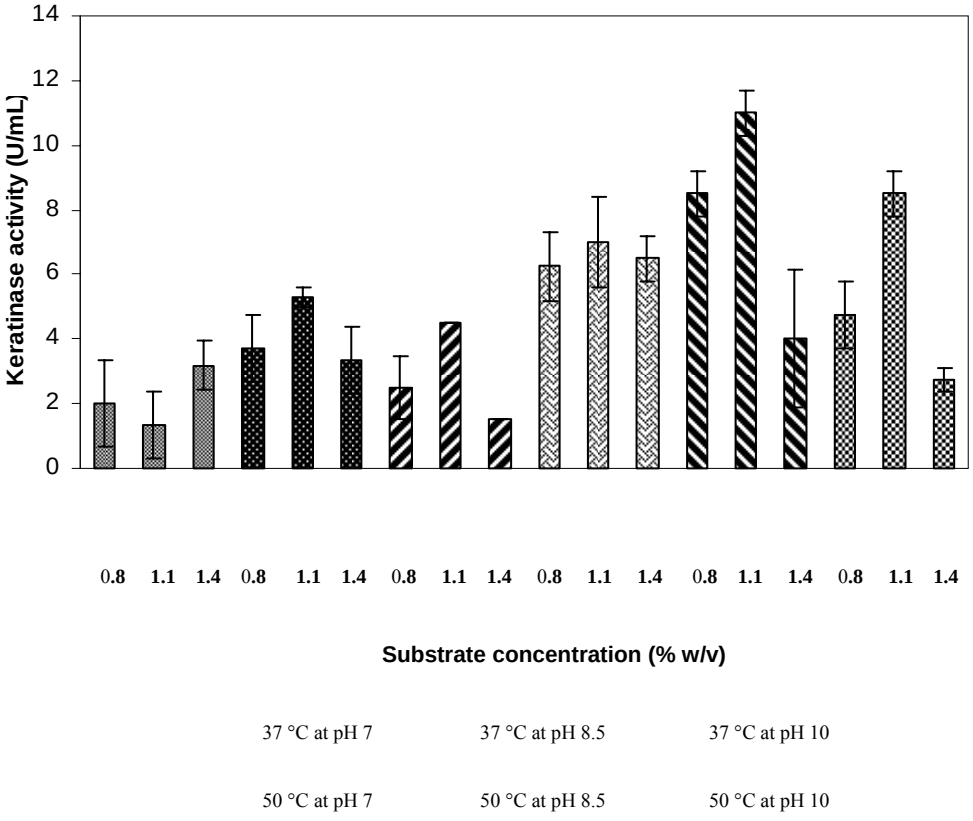


Fig. 4a. MALDI-TOF MS spectrum showing molecular weight of keratinase as ≈ 28KDa.

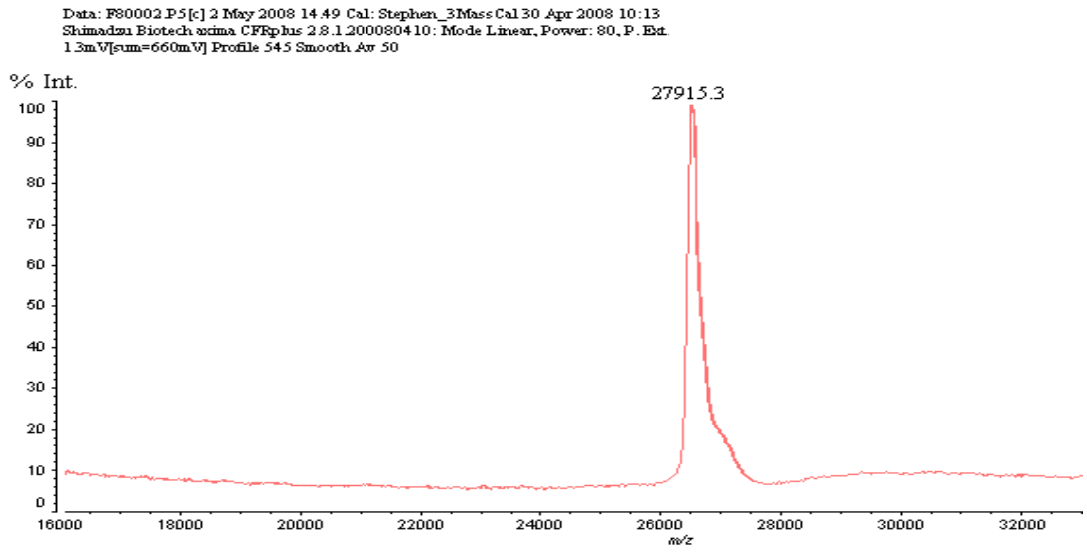


Fig. 4b. SDS-PAGE of the keratinase (Lane 1) ; Lane 2 contains loading buffer only (control) and MWM is the molecular weight marker.

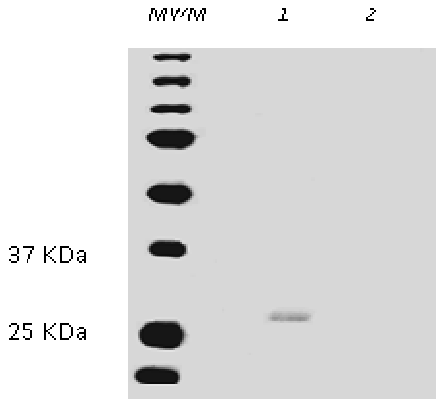


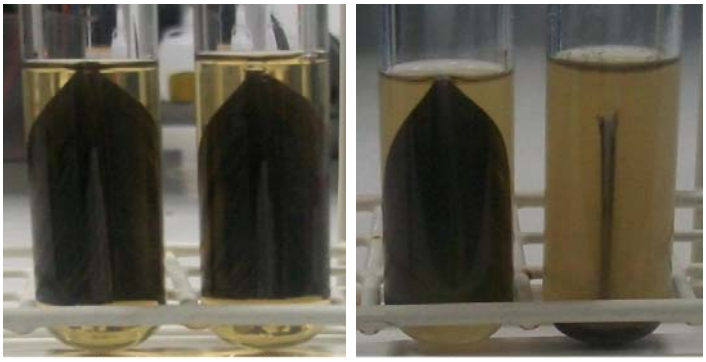
Fig. 5. Degradation of melanised feather by crude keratinase showing the state of feather at 0 and 48 h. In photographs labelled (A), the feathers in test tubes '*a*' and '*b*' were incubated in 10 ml of distilled water and dilute crude keratinase (2 ml in 8 ml of distilled water) respectively. In photographs labelled (B), the feathers in test tubes '*a*' and '*b*' were incubated in 10 ml of denatured crude keratinase (boiled for 30 min) and undiluted crude keratinase respectively.

(A)

a ***b*** ***a*** ***b***

0 h 48 h

(B)



a ***b*** ***a*** ***b***

0 h 48 h